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**13. Abstract** *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Rcl is an undefined protein whose expression can be induced by the oncoproteins c-Myc and HER2/Neu, both of which are heavily implicated in breast tumorigenesis. As such, Rcl may be an important downstream effector of c-Myc and HER2/Neu in breast cancer. To prove this hypothesis, we pursued three lines of research. (i) We studied the potential co-overexpression of Rcl, c-Myc and HER2/Neu in human breast tumor specimens and found Rcl to be overexpressed in one-third of all cases, but seemingly independent of HER2/Neu and c-Myc overexpression. This suggests that Rcl is an oncogene contributing to cancer. (ii) We wished to assess the importance of Rcl for the phenotypic changes observable in tumor cells. To this end, we attempted to knock-down Rcl expression by RNA interference and study the resultant phenotypic changes in tumor cells. However, these experiments could not yet be performed due to our inability to generate effective Rcl siRNA. (iii) To unravel Rcl's mode of action, potential interaction partners have been identified by a yeast two-hybrid screen and a procedure has been developed to isolate Rcl-containg protein complexes amenable for mass spectrometric identification. Thus, the foundation for the identification of Rcl interaction partners has been established.

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## Table of Contents

# Final Report: Rcl, a novel breast cancer oncoprotein?

## Introduction

The Rcl protein is an enigmatic entity that shares no homology to other known proteins and whose molecular modes of action have remained hidden. Originally, the respective Rcl gene was identified as a target gene of the c-Myc oncoprotein. Furthermore, ectopic expression of Rcl induces anchorage-independent cell growth and tumor formation in nude mice, indicating that Rcl plays an important role in oncogenesis [1, 2]. Since c-Myc can also be stimulated by the HER2/Neu oncoprotein [3], Rcl may also be a target of this receptor tyrosine kinase. Indeed, we noted that Rcl mRNA is induced by HER2/Neu, but surprisingly in a manner, at least partially, independent of c-Myc [unpublished results]. As such, Rcl expression can be stimulated by two different pathways triggered by c-Myc or HER2/Neu, two oncoproteins whose frequent overexpression in breast tumors has been correlated with an adverse prognosis [4, 5]. Accordingly, we hypothesized that Rcl represents an important effector of both c-Myc and HER2/Neu in breast tumorigenesis. Here, we wished to test this hypothesis by (i) studying the possible coexpression of Rcl, c-Myc and HER2/Neu in breast tumor specimens, by (ii) demonstrating the importance of Rcl for cell transformation, and by (iii) unraveling potential Rcl interaction partners involved in breast tumorigenesis.

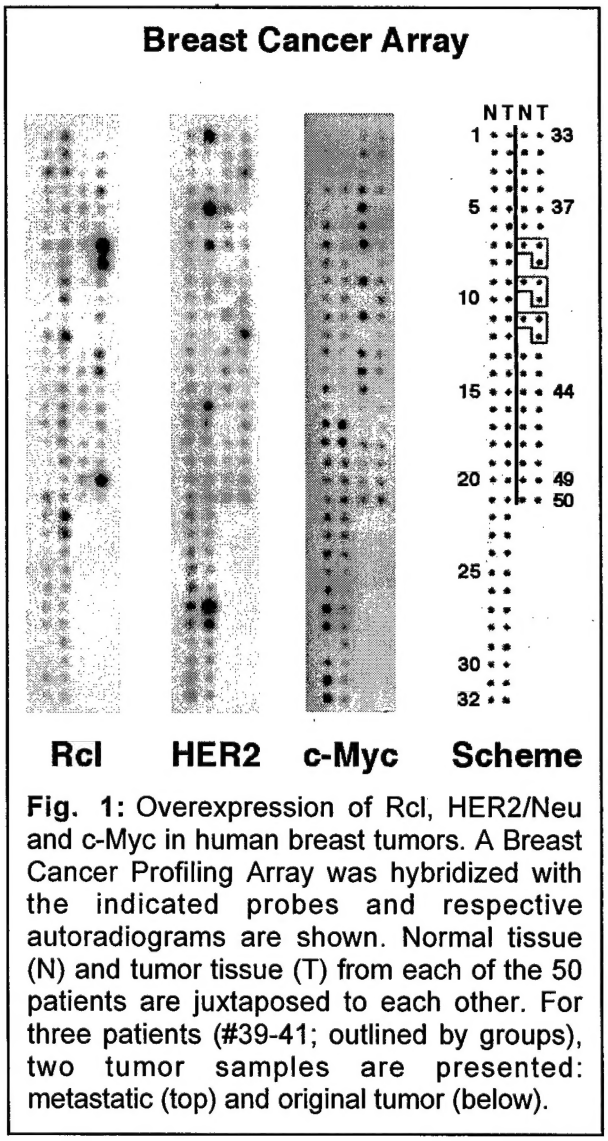


**Fig. 1:** Overexpression of Rcl, HER2/Neu and c-Myc in human breast tumors. A Breast Cancer Profiling Array was hybridized with the indicated probes and respective autoradiograms are shown. Normal tissue (N) and tumor tissue (T) from each of the 50 patients are juxtaposed to each other. For three patients (#39-41; outlined by groups), two tumor samples are presented: metastatic (top) and original tumor (below).

## Body

**Specific Aim 1:** *Study the co-overexpression of Rcl, c-Myc and HER2/Neu in human breast tumor specimens.*

To this end, we utilized a Breast Cancer Profiling Array (Clontech) on which equal amounts of cDNA derived from tumor and

corresponding normal tissue of 50 different breast cancer patients had been spotted. We successively hybridized this array with $^{32}$P-labeled probes derived from human Rcl, c-Myc and HER2/Neu cDNAs. As shown in Fig. 1, Rcl overexpression was noted in approximately one-third of all breast tumors, thereby supporting the hypothesis that Rcl is an oncogene whose overexpression can contribute to breast tumor formation.

With lesser frequency than for Rcl, overexpression of HER2/Neu was also noted, but barely any of c-Myc. Furthermore, there was no significant correlation between Rcl overexpression and that of either HER2/Neu or c-Myc. Thus, we conclude that Rcl overexpression is not due to c-Myc and HER2/Neu overexpression in breast tumors.

Immediately, this raises the exciting question what causes Rcl overexpression in breast tumors. Rcl promoter studies may reveal oncogenic pathways that elicit Rcl overexpression in breast tumors. Another possibility is that Rcl overexpression caused by HER2/Neu and/or c-Myc may be counteracted by an inhibitor of Rcl transcription in the breast. Potentially, such an inhibitor might be tissue-specifically expressed and therefore Rcl overexpression may be correlated to that of HER2/Neu and/or c-Myc in non-breast tumors. Future experiments will address these questions.

**Specific Aim 2:** *Investigate the impact of Rcl on tumor cell phenotype.*

To distinguish whether Rcl overexpression in breast tumors is a consequence of cell transformation or a contributing cause, we wished to analyze the effect of knocking-down Rcl expression on cell transformation by studying phenotypic changes related to growth rate and anchorage-independent growth. We proposed to utilize RNA interference to knock-down Rcl expression. Thus, we cloned various oligonucleotides into the pSUPER vector that directs the production of small-hairpin RNAs known to knock-down gene expression [6]. We generated six different Rcl siRNAs by this way, but none of them proved effective (Fig. 2 and data not shown).

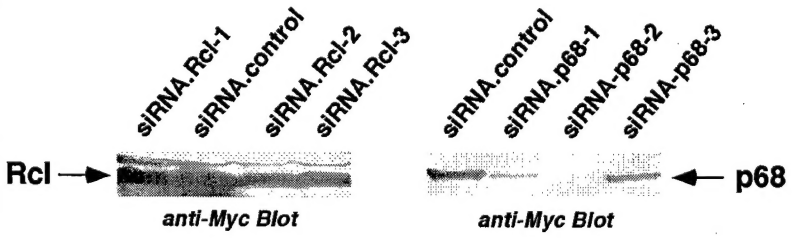


**Fig. 2:** Test of siRNA constructs. Indicated siRNA constructs were transfected into 293T cells together with Myc-tagged Rcl or p68 expression vector. 72 h after transfection, expression levels of Rcl and p68 were analyzed by anti-Myc Western blotting.

As shown in Fig. 2, various Rcl siRNAs did not result in a significant decrease of Rcl protein. This is not due to a principal mistake in our study design, since we were successful in an unrelated project to knock-down another protein, p68, to various extents with similarly produced siRNAs

(Fig. 2). In conclusion, we were unable to perform the proposed experiments of this specific aim due to the inability to generate effective Rcl siRNA.

Rcl gives rise to a relatively small mRNA that seems to have extensive secondary structure such that recognition by siRNA could be inhibited. Alternatives to the knock-down of Rcl expression can be envisaged by utilizing antisense RNA (albeit that may also be hampered by secondary structure formation of Rcl mRNA) or by transfecting/injecting Rcl antibodies. Finally, one could generate hypomorphic or knock-out Rcl alleles in tumor cells via homologous recombination.

**Specific Aim 3:** *Identify potential Rcl interaction partners.*

The Rcl protein sequence gives no clue to its function, and neither has anything been published relating to the molecular mechanisms of its action. To gain insight into the mechanistic function of Rcl, which is important to understand its role in breast tumorigenesis, we pursued to identify Rcl interaction partners by two ways, yeast two-hybrid screen and mass spectrometry of Rcl-containing protein complexes.

For the yeast two-hybrid system, we employed the Matchmaker GAL4 Two-hybrid System 3 (Clontech). Full-length Rcl was fused to the GAL4 DNA binding domain and this construct was then used as a bait for screening a testis library; the bait in itself did not self-activate. A total of $1.6x10^7$ colonies were screened and $1.6x10^4$ putative positives were obtained. This was an unexpected high number, since one would normally expect ~100-fold less. Nevertheless, we picked 1300 putative colonies and selected those 200 which could even grow under the most stringent selection conditions, which included 50 mM 3-AT. DNA was obtained from those 200 colonies and utilized to re-check interaction with the Rcl bait, but also with three unrelated baits in yeast. 99 clones were thereby excluded, since they showed unspecific interaction with other baits. The remaining 101 clones were DNA sequenced, but we found that only 2 clones showed in-frame fusions of open-reading-frames to the activation domain in the prey plasmid. Those two clones were kinesin family member 2C and an unknown bHLH protein, Math6. Currently, we pursue to obtain full-length cDNAs of these two clones so that we can reconfirm their interaction in mammalian cells with Rcl employing co-immunoprecipitation assays.

We do not know why the Rcl bait gave so many positives in the yeast two-hybrid screen, and therefore we do not know if at all this method would be useful. Should we be able to reconfirm in mammalian cells the interaction of Rcl with the two positive clones (kinesin family member 2C and Math6), we might go back and analyze more than the 1300 out of the 16000 original putative positive clones.

As an alternative to the yeast two-hybrid screen, he have proposed to fuse an HA-tag onto Rcl, transfect 293T cells with this construct and then isolate Rcl and associated proteins by anti-HA immunoprecipitation and identify them by mass spectrometry. This method resulted in a plethora

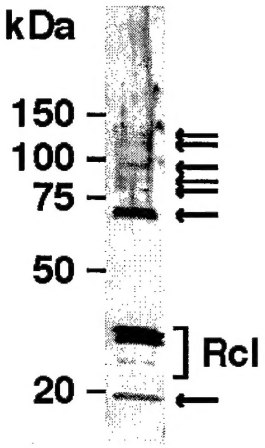


**Fig. 3:** Isolated Rcl protein complexes after tandem-affinity purification. A silver-stained gel shows copurified proteins (see arrows).

of proteins being co-immunoprecipitated, indicating that a single purification step is not sufficient to specifically isolate Rcl interaction partners. Therefore, we introduced two further tags into HA-Rcl: a CBP (calmodulin binding peptide) and an SBP (streptavidin binding peptide) tag. We then performed a tandem-affinity purification in order to enrich Rcl interaction partners: the SBP tag allows purification on a streptavidin column and mild elution with biotin, whereas the CBP tag allows purification on a calmodulin column and mild elution with EGTA [7]. Combined with a mild lysis procedure of transiently transfected mammalian 293T cells that express the SBP-CBP-HA-Rcl fusion protein, these consecutive affinity purifications resulted in the isolation of enriched Rcl-containing protein complexes. These complexes have been resolved by SDS-PAGE. Fig. 3 shows that several protein bands were detectable, and even more so upon applying more sensitive detection. Therefore, the degree of purity is not sufficiently high enough to analyze these protein complexes by mass spectrometry. which is why we are currently performing additional anti-HA immunoprecipitations to obtain highly purified Rcl-associated proteins. Once we have combined the tandem-affinity purification (which can be regarded as a preclearing step) with the anti-HA immunoprecipitation, we will obtain pure enough samples that will be amenable for mass spectrometric analysis thereby revealing the identity of Rcl-associated proteins.

Once we have identified those unknown proteins, we will reconfirm their interaction with Rcl by coimmunoprecipitation experiments and then delve into the analysis how these interaction partners, together with Rcl, contribute to breast tumorigenesis.

## Key Research Accomplishments

- Unraveled that Rcl is overexpressed in one-third of all human breast tumors
- Shown that Rcl overexpression in breast tumors does not correlate with HER2/Neu and c-Myc overexpression
- Established a procedure to purify Rcl interaction partners that will allow their identification by mass spectrometry

## Reportable Outcomes

Funding applied for based on work supported by this award:
Renewal of NIH grant R01 CA085257, Dysregulation of the Transcription Factor ER81 (revised version supposed to be resubmitted by Nov. 1, 2004). One specific aim makes use of the fact that Rcl is overexpressed in human breast tumors, the reason of which we would like to study in this grant proposal.

Training of postdoctoral fellow:
The payment of Dr. Sook Shin's salary has been supported by this award.

## Conclusions

The main conclusion is that Rcl is overexpressed in ~1/3 of all human breast tumors, yet the reason why it is has remained unresolved. Furthermore, the purification of protein complexes containing Rcl will allow the elucidation of its interaction partners, which will eventually give us clues to how Rcl contributes to breast tumorigenesis. Since Rcl knock-down experiments by siRNA have failed, alternatives such as conditional knock-out models should be developed. Also, transgenic mice overexpressing Rcl in the breast should provide insight into the role of Rcl during breast tumorigenesis.

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